

Molecular Characteristics and Clinical Relevance of Cytotoxin-Associated Genes A and E of *Helicobacter pylori* from Patients with Gastric Diseases

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Abstract

Background: *Helicobacter pylori* colonizes about a half of the world's population. It possesses several genes that associated with virulence, among which are cytotoxin-associated antigen (*CagA*) and cytotoxin-associated gene E (*CagE*) genes within the *cag* pathogenicity island. These genes encode for proteins involved in the pathogenicity of the bacteria. **Objective:** This study aimed to investigate the prevalence of *CagA* and *CagE* genes among *H. pylori* isolates from patients with different gastric pathologies and for phylogenetic analysis of the isolated bacteria according to gene sequence. **Materials and Methods:** A total of 104 gastric biopsies were collected for patients suffering from different gastric pathologies. DNA was extracted from these biopsies, and a conventional polymerase chain reaction (PCR) was performed to amplify the gene fragments corresponding *CagA* and *CagE* genes using specific primers. PCR product of selected samples of positive for *CagE* was undergone direct sequencing. The result of sequences was aligned with reference sequences in National Center for Biotechnology Information (NCBI), and the phylogenetic tree was constructed. **Results:** Out of 104 isolates of *H. pylori*, 89/104 (85.58%) were found to have either *CagA*, *CagE*, or both genes. The frequencies of *CagA*, *CagE*, and coexistence of both genes were 71.15%, 46.15%, and 31.73%, respectively. The phylogenetic tree revealed two main clades, one of which involved isolates 6 and 9 as separated isolates and another clade involved all other isolates. The isolate 4 clustered with AY153111.1 and AP014523.1, the isolate 3 clustered with AY153124.1, the isolate 5 clustered with LC339073.1 and LC339017.1, the isolates 7 and 11 clustered with EU090726.1, the isolate 2 clustered with AB191082.1, the isolate 10 clustered very close to LC339004.1 and less close to LS483488.1, while the isolate 1 clustered with AP017334.1. **Conclusion:** *CagA* and *CagE* genes are highly prevalence among *H. pylori* isolate from gastric pathologies from Iraqi patients.

Keywords: Cytotoxin-associated antigen, cytotoxin-associated gene E, *Helicobacter pylori*, phylogenetic analysis

INTRODUCTION

Helicobacter pylori, a spiral, Gram-negative, microaerophilic bacterium, colonizes at least half of the world's population and is recognized as a major cause of chronic gastritis (CG) and peptic ulcer disease (PUD) and as an important risk factor for gastric cancer (GC).^[1] On the basis of various epidemiological studies, *H. pylori* was classified as a Class I carcinogen in humans by a working group of the World Health Organization International Agency for Research on Cancer.^[2] Infection rates vary among the developed and developing countries of the world. *H. pylori* infection is on a steep decline in most of the western countries mainly due to the success of combination

therapies and improved personal hygiene and community sanitation to prevent reinfection. However, the situation is not improving in many of the developing countries due to failure of treatment regimens and emergence of drug resistance.^[3]

One of the most distinctive features of *H. pylori* is the genetic diversity between clinical isolates obtained from

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different patient populations. Most *H. pylori* isolates can be discriminated from each other by DNA profiling^[4] or sequencing of certain genes mainly due to a high degree of sequence divergence between orthologs (3%–5%).^[5]

H. pylori infection is usually acquired during childhood, where transmission occurs predominantly within families.^[4] A couple of recent studies demonstrated the possible coexistence of a large array of clonal lineages within *H. pylori* populations that are evolving in each individual separately from one another.^[5] It is, therefore, probable that through this semi-vertical transmission of *H. pylori* strains, there are distinct sets of *H. pylori* genotypes colonizing different human populations. With different strains evolving separately of one another and the fact that *H. pylori* is a genetically diverse organism, distinct genotypes have been found to be associated with particular geographic regions.^[6] The cytotoxin-associated antigen (*CagA*) gene of *H. pylori* was assumed as partially responsible for eliciting signaling mechanisms that lead to the development of gastric pathologies. Based on the carriage of a functional *CagA* as a marker for the *cag* pathogenicity island (*cag* PAI), the *H. pylori* species is divided into *CagA*-positive and *CagA*-negative strains. The *CagA*-positive strains were thought to be associated with higher grades of gastric or duodenal ulceration and are more virulent than the *CagA*-negative strains;^[7] however, such assumption was not confirmed. Furthermore, some epidemiological studies have demonstrated the roles of *CagA*-positive *H. pylori* in the development of atrophic gastritis, PUD, and GC.^[8]

Cytotoxin-associated gene E (*CagE*) is one of the marker genes in *cagI* of the *cag* PAI. It is essential for *CagA* translocation and phosphorylation.^[9] Some reports indicated that the presence of the *CagE* gene has been associated with a bad clinical outcome, especially in developed countries.^[10]

In Iraq, there is no previous study of phylogenetic analysis of *H. pylori*. Therefore, the current study aimed to investigate the prevalence of *CagA* and *CagE* genes among *H. pylori* isolates from patients with different gastric pathologies and for phylogenetic analysis of the isolated bacteria according to gene sequence.

MATERIALS AND METHODS

Patients and samples

This is a cross-sectional study including a total of 104 patients suffering from gastric diseases, who underwent esophagogastroduodenoscopy (EGD) and examination at Gastrointestinal Tract and Liver Diseases Institute, Baghdad, from December 2017 to September 2018. Two gastric tissue biopsies were obtained from the antral part of the stomach during gastrointestinal endoscopy. The first one was sent to a histopathological laboratory while the second was placed in 1 ml of normal saline and preserved at –20°C for molecular analysis.

Histopathology examination results were taken from patient record. According to these results, patients were grouped to CG, acute gastritis (AG), and gastric ulcer (GU).

DNA was extracted from these biopsies using a commercial ready kit (The Wizard® Genomic DNA Purification Kit/Promega).

The present study was approved by the Ethical Committee of the College of Medicine/Al-Nahrain University.

Measurement of extracted DNA concentration and purity

A NanoDrop (UVS-99/ACT Gene/USA) was used to evaluate the concentration and purity of the extracted DNA.

Conventional polymerase chain reaction to detect *Helicobacter pylori*

Conventional polymerase chain reaction (PCR) was performed to amplify the gene fragments corresponding *CagA* (350 bp) and *CagE* (508 bp) genes using specific primers *CagA* forward (GATAACAGGCAA GCTTTTGGAGG) and reverse (CTGCAAAAG ATTGTTTGGCAG) and *CagE* forward (TTGAAAACCTTCAAGGATAGGATAGAGC) and reverse (GCCTAGCGTAATATCACC ATTACCC).^[11,12] GoTaq® Green Master Mix is a premixed ready-to-use solution containing bacterially derived Taq DNA polymerase, deoxynucleoside triphosphates, MgCl₂, and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. Four microliters of template DNA and 2 µl of each primer (forward and reverse) were added to the master mix tube. The final volume was adjusted to 50 µl with free nuclease distal water. The mixture was then centrifuged for 10 s and put in thermocycler (Hybaid/UK) which was previously programmed with the following conditions: 94°C, 5 min one cycle (94°C, 30 s and 46°C, 30 s and 72°C, 30 s 40 cycles) 72°C, 10 min one cycle.

DNA sequencing

The PCR products of *CagE* gene were directly sequenced using Big Dye terminator method (Macrogen/Korea). The obtained sequences were aligned with normal sequence from GenBank.

Statistical analysis

The Statistical Package for the Social Sciences version 25 (SPSS, IBM, Chicago/USA) was used for all statistical analyses. Continuous variables were expressed as mean ± standard deviation and analyzed with ANOVA. Binomial variables were expressed as frequency and percentage and analyzed with Chi-square. Phylogenic relevance between local isolates and reference isolates and within local isolates was calculated using Mega 7 software/ Pennsylvania State University/USA with 1000 bootstrap replicates. *P* < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics and frequencies of different gastric diseases

This study included a total of 104 patients who underwent EGD and found to be *H. pylori* positive. Three major categories

of gastric illness were represented in those patients based on histopathological examination. These categories were CG (49, 47.11%), AG (49, 47.11%), and GU (49, 47.11%), as shown in Figure 1.

Molecular assay

Cytotoxin-associated antigen gene gel electrophoresis

Gel electrophoresis of PCR product for *CagA* gene is presented in Figure 2. The PCR product was 350 bp.

Cytotoxin-associated Antigen gel electrophoresis

Gel electrophoresis of PCR product for *CagE* gene is presented in Figure 3. The PCR product was 508 bp.

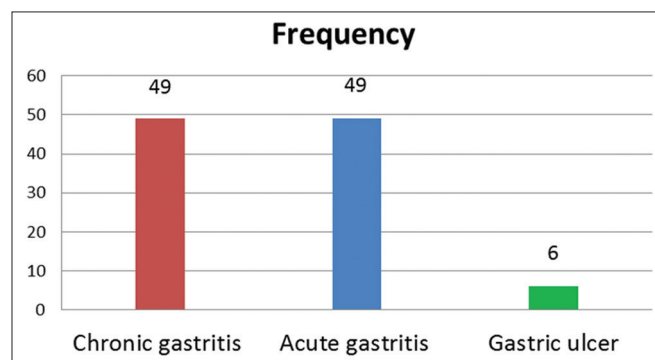


Figure 1: Frequencies of gastric clinical characteristics

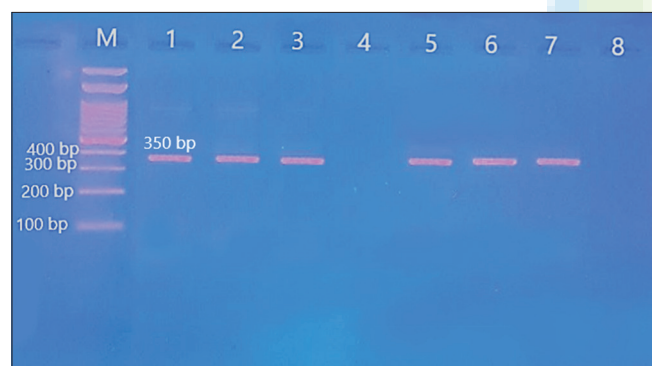


Figure 2: Gel electrophoresis of polymerase chain reaction product for cytotoxin-associated antigen gene. Molecular ladder (M), lanes (1, 2, 3, 5, 6, and 7) are positive for cytotoxin-associated antigen gene with fragment length of 350 bp, lanes 4 and 8 are negative for cytotoxin-associated antigen gene

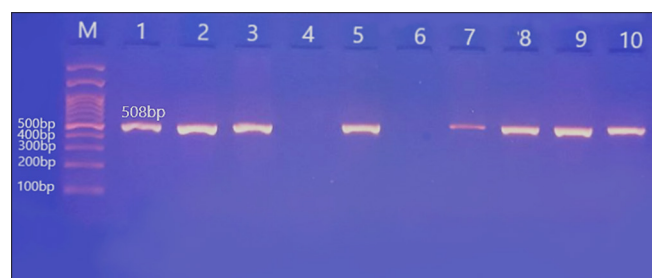


Figure 3: Gel electrophoresis of polymerase chain reaction product for cytotoxin-associated gene E. Molecular ladder (M), lanes (1, 2, 3, 5, 7, 8, 9, and 10) are positive for cytotoxin-associated gene E with fragment length of 508 bp, lanes 4 and 6 are negative for cytotoxin-associated gene E

Prevalence of cytotoxin-associated antigen, cytotoxin-associated gene E, or both genes in *Helicobacter pylori* from patients with gastric illnesses

Out of 104 isolates of *H. pylori*, 89/104 (85.58%) were found to have either *CagA*, *CagE*, or both genes. The frequencies of each gene among the total isolates were 74/104 (71.15%) for *CagA*, 48/104 (46.15%) for *CagE*, and 33/104 (31.73%) for both genes.

Genetic analysis of *Helicobacter pylori*

Alignment of *Helicobacter pylori* sequences with reference sequences

The PCR products from 11 samples of *H. pylori* positive were sent abroad to Macrogen Company in South Korea for direct sequencing. As *CagE* gene is a protein-coding gene, the nucleotide sequences of this gene were translated into the corresponding amino acid using EMBOSS Transeq available online at https://www.ebi.ac.uk/Tools/st/emboss_transeq/. As well, the ten reference isolates were AP017334.1, AB191082.1, AY153111.1, AP014523.1, AY153124.1, LC339073.1, LS483488.1, LC339017.1, EU090726.1, and LC339004.1, translated by this program. BioEdit software, version 6 (Carlsbad/ CA, USA) was used to align and compare the different amino acid sequences between local isolates and reference sequences [Figures 4 and 5].

The nucleotide sequences of this gene were translated into the corresponding amino acid using EMBOSS Transeq.

Phylogenetic tree

Furthermore, the genetic relatedness of the local isolates to the selected reference isolates was analyzed with Mega 7 software (Pennsylvania State University/USA) through the construction of phylogenetic tree [Figure 6]. The phylogenetic tree revealed two main clades, one of which involved isolates 6 and 9 as separated isolates and another clade involved all other isolates, as well as the reference isolates, isolate 8 although clustered within the other isolates; however, it had no closest reference isolates. The local isolate 4 clustered with AY153111.1 and AP014523.1, the isolate 3 clustered with AY153124.1, the isolate 5 clustered with LC339073.1 and LC339017.1, the isolates 7 and 11 clustered with EU090726.1, the isolate 2 clustered with AB191082.1, the isolate 10 clustered very close to LC339004.1 and less close to LS483488.1, while the isolate 1 clustered with AP017334.1.

DISCUSSION

The *CagA*, as a marker for the presence of the *cag* PAI, is one of the best-studied virulence factors for *H. pylori*, and the frequency of *CagA*-positive isolates has been reported as 74% in the current study. Globally, *CagA* gene was reported in about 25%-60% of *H. pylori* isolates in Bahrain and Jordan, and 60%-80% in other countries such as Taiwan, Malaysia, and India.^[13] According to a study, the *CagA*-positive genotype varied geographically from 44% to 94% in Iranian populations.^[13] On the other hand, in a study from Turkey, Erdoğan *et al.* reported a *CagA* positivity rate of 65.5%.^[14]

Hamad, *et al.*: *CagA* and *CagE* genes of *Helicobacter pylori*

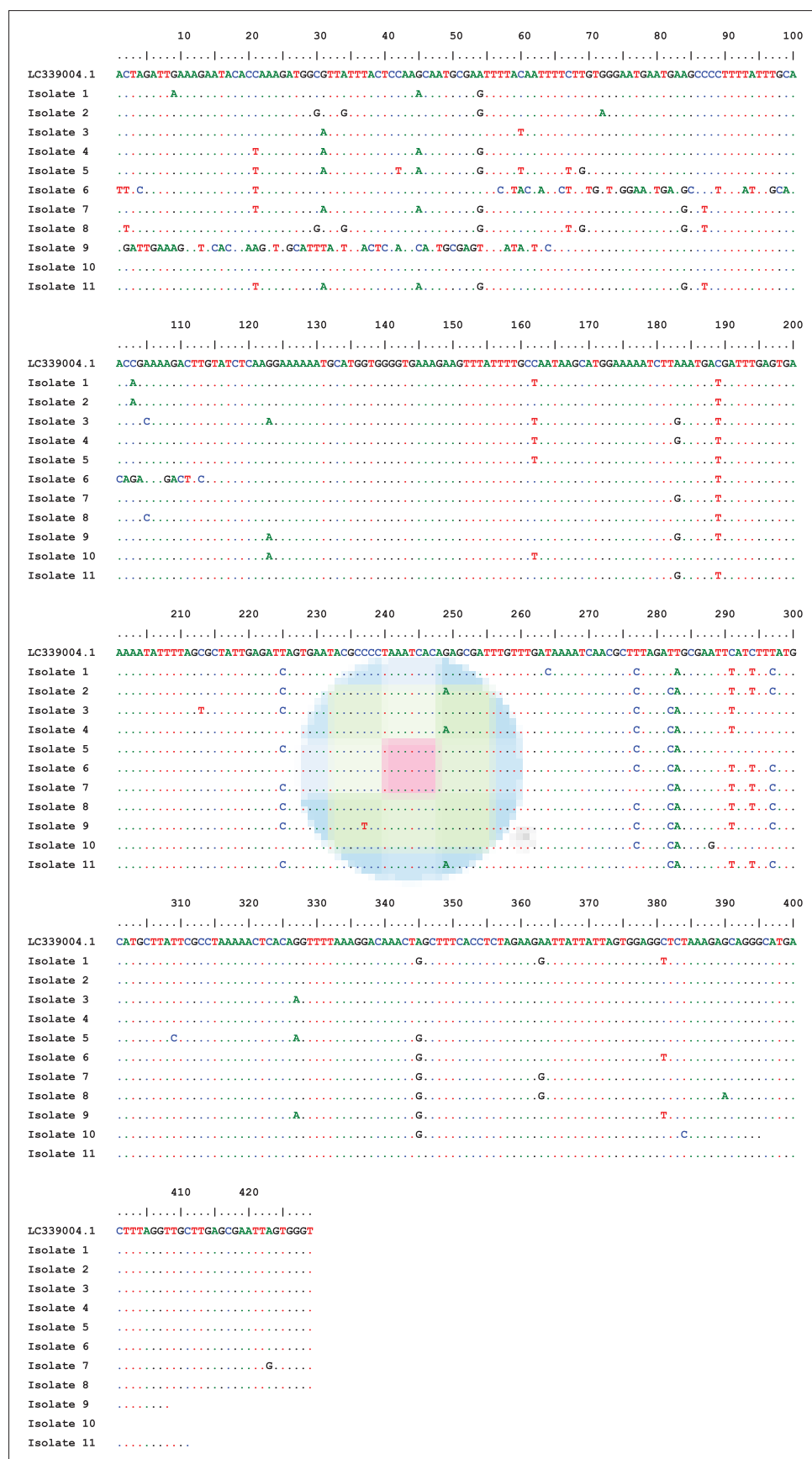


Figure 4: Alignment of 11 *Helicobacter pylori* local isolates with 1 reference isolate from GeneBank (indicated by its accession number) using BioEdit software

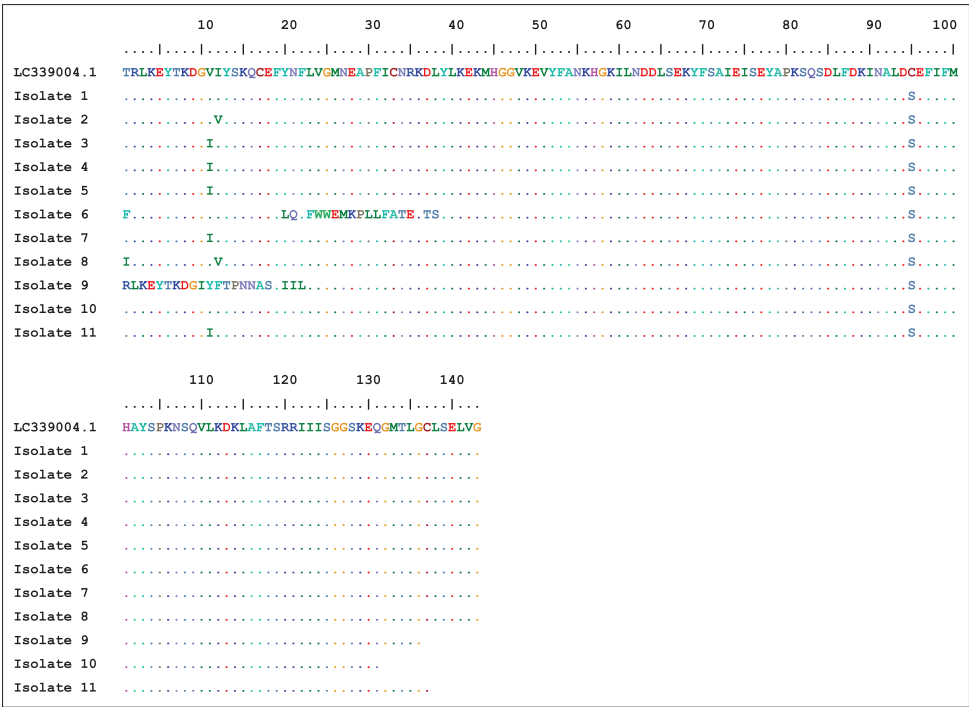


Figure 5: Alignment of 11 *Helicobacter pylori* local isolates with 1 reference isolate from GeneBank (indicated by its accession number) using BioEdit software

The prevalence of *CagA*-positive strains of *H. pylori* differs in various parts of the world.^[15] For example, Kadi *et al.*^[16] reported a prevalence rate of 81.7%, while in Tunisia,^[17] Egypt,^[15] Palestine^[18] and Iran,^[19] the prevalence rates of the *CagA* gene were 61.6%, 65.2%, 65.9%, and 68.7%, respectively. In Russia, Cyprus^[20-22] and East to South Asian countries,^[1,23,24] the reported prevalence was 85%, 42.5%, and 90%, respectively.

In another study in Brazil, the frequency of *CagE* gene was 53.2%, and most of the *H. pylori* strains *CagA* positive were associated with *CagE* positive (62.3%).^[25]

CagE was found more commonly in patients with GC than in the other patient groups (93.8%; 15/16). The combination of the *CagA* and *CagE* genes was detected in 87.5% (98/112) of the patients.^[23]

In another study in the USA, the *CagE* gene, also within the PAI and shown to stimulate the production of several cytokines from infected epithelial cells, was found in 62% of with *H. pylori* and in 95% of *CagA*-positive *H. pylori*, where all *CagE*-positive *H. pylori* were also *CagA* positive.^[18]

The presence of the *CagE* gene was also reported to vary in different geographic regions and/or ethnic groups (*e.g.*, 64% in the US, 71% in the UK, 70% in Malaysia, 88% in Thailand, 28% in Turkey, and 77% in India).^[1,23,26-29] The prevalence of the *CagE* gene in studied isolates from both countries was close to each other (51% in Iranian vs. 53% in Afghani isolates), and different with other countries, another finding was different with a very limited report on only three Afghani samples.^[30]

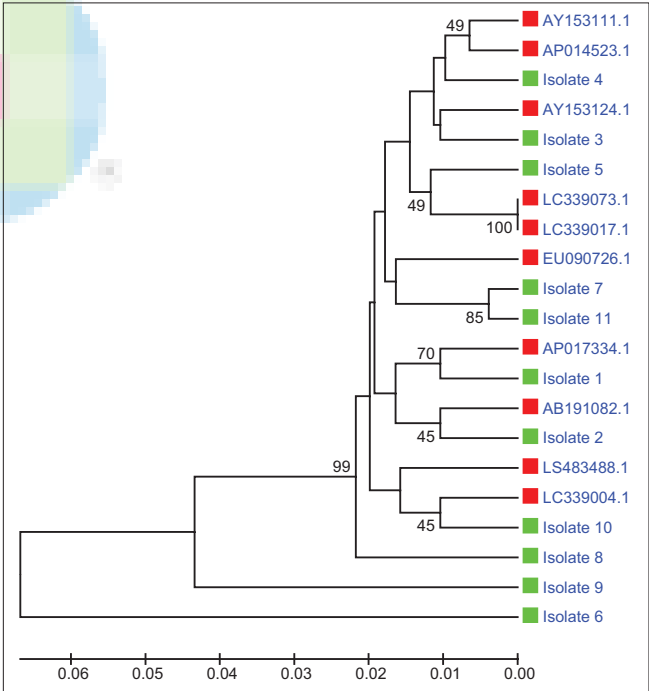


Figure 6: Phylogenetic tree for cytotoxin-associated gene E constructed by the 11 local isolates from gastric tissue biopsies and 10 reference isolates from GeneBank. Model of the tree was statistically supported by bootstrapping with 1000 replicates. Bootstrap values below 30% are not shown. Current isolates are indicated with green square

Frequencies of different gastric diseases of patients who underwent EGD were 104 and they were found to be *H. pylori* positive and classified into three major categories of gastric

illness were recognized based on histopathological examination. The categories were CG (49, 47.11%), AG (49, 47.11%), and GU (6, 5.7%).

Compared to other studies, more than 90% of people infected with *H. pylori* experienced varying degrees of gastritis, with moderate gastritis (51.10%) having the highest incidence.^[31] In the study conducted by Hashemi *et al.* in southern Iran (2006), 70% of people infected with *H. pylori* suffered from CG, and in another study in Saudi Arabia, this rate was 60%.^[32,33]

A study was also showed that there was a direct relationship between the severity of *H. pylori* infection and the degree of CG. Hence, if the level of colonization was mild, the chance of severe CG among the participants was only 0.57% while this chance was about 28% among those with severe colonization.^[31]

In a study conducted by Ardakani and Mohammadizadeh in Isfahan on 272 samples of gastric biopsy, there was no relationship between the density and volume of *H. pylori* and the severity of CG activity.^[34] In a study by Yakoob and Hussainy in Pakistan, there was a relationship between the intensity of *H. pylori* colonization and CG activity.^[35]

The findings of the study showed that there was a dose-response relationship between *H. pylori* colonization and gastritis such that in mild colonization, only 20.63% of the samples had severe gastritis, while in intense colonization of *H. pylori*, 43.75% of the samples had severe gastritis.^[31] In a study by Alagoz *et al.* in Turkey (2002), it was shown that there was a relationship between the level of colonization and gastritis.^[36]

This relationship was not found in the study conducted by Park *et al.* in Korea, which could be due to the genetic differences, nutritional habits, and environmental factors between the two study populations.^[37]

Most isolates (2–5, 7, 8, and 11) differed only in two amino acids, while 1 and 10 isolates differed in only one amino acid, which the majority of nucleotide variations of isolates (1–5, 7, 8, 10, and 11) were synonymous. The only exception are isolates 6 and 9 which they had 19 and 22 substitutions, respectively. Furthermore, the two isolates differed between each other which may indicate the presence of new alleles for *CagE* gene and reflects the genetic diversity of the gene among different isolates of *H. pylori*.

Phylogenetic analysis revealed the wide diversity of Iraqi isolates of *H. pylori*. Unfortunately, there is no local reference isolate registered in NCBI. Therefore, the results of the sequences were compared with international isolates. Three of local isolates (3, 5, and 10) had clustered with an Indonesian isolate, while other three of local isolates (4, 2, and 1) had clustered with a Japanese isolate, and other two of local isolates (11 and 7) had clustered with a Chinese isolate. The phylogenetic tree revealed two main clades, one of which involved isolates 6 and 9 as separated isolates and another clade involved all other isolates, as well as the reference isolates,

isolate 8 although clustered within the other isolates; however, it had no closest reference isolates.

The vast majority of the isolates in the current study had closed with Asian isolates (three with an Indonesian isolate, three with a Japanese isolate, and two with a Chinese isolate). This indicates the importance of modern communications and transporting in bacterial transfer between different countries. However, it is worthy to mention that none of the local isolates were identical to any of the reference isolate which implies a unique molecular structure of the local isolate.

Furthermore, the local isolate 4 also had closed clustering to a Spanish isolate (AY153111.1). On the other hand, the isolate 10 had less close of clustering to the United Kingdom isolate (LS483488.1), which reflects the high levels of reciprocal travels between Iraq and other European countries (Spanish and United Kingdom).

CONCLUSION

From this study it can conclude that a highly prevalence of *CagA* and *CagE* genes is recorded among *H. pylori* isolates recovered from gastric pathologies from Iraqi patients.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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